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REVIEW

Cellular and Molecular Aspects of Embryonic Induction

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ABSTRACT—A homogeneous protein, which induces mesodermal and endodermal tissues in amphibian gastrula ectoderm ("vegetalizing factor") has been isolated from chicken embryos. Inducing factors with similar chemical properties have been found in the *Xenopus* XTC-cell line and in calf kidney. The factors belong to evolutionary conserved proteins, which may also have regulatory functions in later differentiation processes or the maintenance of differentiation. They are related to the TGF-β protein superfamily. Members of this and of the FGF protein family induce mesodermal tissues. In early embryos mesoderm inducing factors show a graded distribution. Masked maternal neural inducing factors are in part activated after the cleavage stages. They have been partially purified from *Xenopus* embryos. We could show that phorbolester evokes neural differentiation, suggesting a signal transduction mechanism which may include phospholipases and protein kinases.

INTRODUCTION

Amphibian eggs and embryos have been widely used to study the development of vertebrates. Early stages which can be handled with relative ease, are endowed with a high regulatory capacity. They are favorite objects for the study of tissue determination and induction. Determination has been defined as a process which initiates a specific pathway of development by singling it out from various possibilities for which the system is competent [1]. In very early stages determination is not yet definitive (except for endoderm) and can be changed by inductive tissue interactions. Dorsal ectoderm isolated before gastrulation forms epidermis like cells as does ventral ectoderm [2]. In 1924 Spemann and Hilde Mangold discovered that the ectoderm can be induced by the presumptive mesoderm [3]. When the induced dorsal ectoderm is isolated after gastrulation it forms the different parts of the nervous system. The induction of the nervous system is not an all or none, but a progressive process. Isolation experiments [4] and grafting experiments [5] have shown that at successive stages of gastrulation neural differentiations are obtained in the following order: Neural crest derivatives, archencephalic (forehead) and deuterencephalic (hindhead) structures.

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Test of inducing activity on omnipotent gastrula ectoderm

The inducing activity of tissues or isolated factors can be tested on omnipotent gastrula ectoderm. The differentiation of ectoderm can up to the early gastrula stage still be channelled into other pathways by the addition of inducers. In the implantation method devised by Mangold [6, 7] a piece of tissue or a pellet which contains inducing factors is implanted through a slit in the ectoderm into the blastocoelic cavity of an early gastrula of *Triturus* or *Ambystoma*. By the gastrulation movements the implanted tissue is brought into contact with the ventral ectoderm (Fig. 1). Purified factors

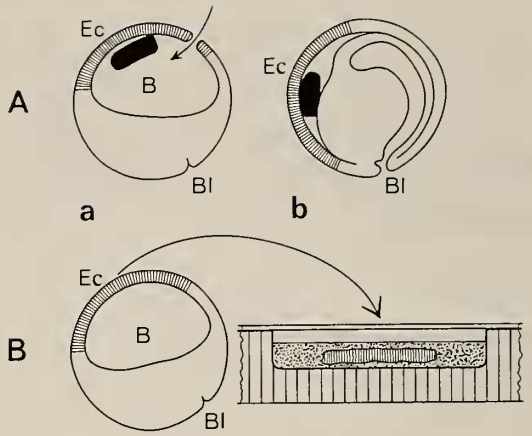


Fig. 1. Test of inducing factors. A. Implantation method a) Insertion of the implant into the blastocoelic cavity of an early gastrula, b) Position of the graft after gastrulation, facing the ventral ectoderm. B. Test on isolated ectoderm in solution. Ec= ectoderm; B=blastocoelic cavity; Bl=blastopore. From Knöchel *et al.*, Blut, 59: 208 (1989), Springer-Verlag, Berlin.

can be tested by the implantation method in different dilutions, if the inducing substance is mixed with a non-inducing protein (i.e. γ-globulin) before the pellet for implantation is prepared. *Xenopus* gastrulae cannot be used for the implantation method [8] due to the different architecture of *Xenopus* embryos. Inducing factors can be tested in solution on isolated ectoderm of *Xenopus, Triturus* or *Ambystoma* embryos [7, 9, 10]. Bovine serum albumin is added to the solution to prevent the adsorption of the small amount of highly purified inducing factors to glass or plastic surfaces [11]. The test on isolated ectoderm has the advantage that *Triturus* and *Ambystoma* as well as *Xenopus* embryos which are available all the year round can be used, but the disadvantage that all induced explants must be examined histologically. Biochemical markers are at present only available for a limited number of tissues.

The response of ectoderm from different species especially to neural inducers is different. *Ambystoma* ectoderm is most susceptible to neural induction. Isolated ectoderm cultivated in a physiological salt solution forms neural tissue without the addition of an inducing factor (so called autoneuralization). *Triturus alpestris* ectoderm does on the contrary not form any neural tissue at physiological salt concentration, but forms neural tissues when the Na^+ ion concentration is increased 1.5 fold [7]. Xenopus ectoderm is the least susceptible and does not form neural tissues at an 1.5 fold increased salt concentration. A strong autoneuralization, however, has been observed if the *Xenopus* ectoderm is dissociated into single cells and reassociated after a lag period [12].

Neural induction and neural inducing factors

The temporal changes of the neural inducing capacity of the presumptive dorsal mesoderm as well differences in the regional inductive capacity of the invaginated mesoderm (the archenteron roof) have been extensively investigated. The experiments have been carried out on *Triturus* and *Ambystoma* embryos which are more suitable for this type of investigation than the smaller and more rapidly developing *Xenopus* embryos [review 14]. The presumptive dorsal mesoderm of early cleavage stages induces neural tissues at a very low rate. The inducing activity starts at the morula stage [13]. Saxén and Toivonen [15] discovered that the simultaneous implantation of a mostly forehead inducing heterologous tissue (guinea pig liver) and a mostly mesoderm inducing tissue (guinea pig bone marrow) induced preferentially forehead, hindhead or neural tube depending on the proportions of neural and mesodermal inducers. This did lead to the theory that a double gradient of a neuralizing and a mesodermalizing agent are in the embryo responsible for the induction of the different regions of the nervous system. By other experiments ("the fold method") Nieuwkoop *et al.* [16] came to the conclusion that in neural induction a wave of activation (resulting in archencephalic structures) is followed by a wave of transformation (resulting in deuterencephalic structures). Attempts by Tiedemann *et al.* [17] to purify a deuterencephalic (hindhead) inducer led to the separation of a neural-archencephalic (forehead) and a mesoderm inducing fraction. Combination of the two fractions in different proportions did lead to hindhead and neural tube induction [18]. Toivonen and Saxén combined dispersed cells of the mesoderm and the forebrain anlage of

gastrulae in different ratios [19, 20]. They observed that a larger proportion of mesodermal cells causes formation of more caudal parts of the central nervous system. The ability of cells of the forebrain anlage to be transformed to more caudal parts of the brain is lost after about 10-12 hr, the transforming ability of the mesoderm lasts somewhat longer [21]. Isolated dorsal ectoderm of early *Triturus* gastrulae (which in normal development is induced to the nervous system) as well isolated ventral ectoderm can be induced to neural structures [22, 23]. In *Xenopus* the ventral ectoderm is, however, induced less frequently. This could be due to an earlier loss of competence in the ventral ectoderm but can not be considered as neural "predetermination" of dorsal ectoderm, which as ventral ectoderm forms neural tissues only after a neuralizing stimulus. The homeotic gene XHox 6 which is expressed early in the development of the neural plate and restricted to the middle and posterior part of the neural axis was used as a marker for neural induction in these experiments [24]. An antigen related to the *Drosophila* homeotic gene invected (which is involved in segmentation) is expressed in the anterior neural plate [25].

The experiments on the transmission of the neural stimulus and the chemical nature of neural inducing agents are described in an excellent review by Saxén [26] and will only briefly be mentioned. Saxén and Toivonen have shown that the separation of the inducing mesoderm and the reacting ectoderm by nucleopore filters with pore diameters down to 0.05 μ did not prevent neural induction. Cytoplasmic bridges were not observed by electronmicroscopy, so that direct cell contacts in the transfilter experiments can be ruled out [27, 28]. The induction depends on short range interactions. Tacke and Grunz [29] observed that a close juxtaposition of the ectoderm and the inducing chorda mesoderm (distance of plasma membranes of opposite cells less than 50 nm) is needed for neural induction. This is correlated with an increase of the number of coated pits, a feature of receptor-mediated endocytosis, in the ectoderm. Connecting cell projections between ectoderm and mesoderm but no cytoplasmic bridges, which could allow a free transfer of inducing factors through intercellular channels, are formed during the mid-gastrula stage [30].

The neural inducing activity of the blastoporal lip is diminished when the secretion of the factor from the blastoporal lip is impaired by treatment with actinomycin D or cycloheximide, substances which could inhibit the synthesis of components of the export system [31]. The factor may be secreted by a mechanism similar to that found in oocytes. Oocytes can secrete proteins which are injected or synthesized on foreign m-RNA. The proteins are probably sequestered into vesicles and exported by exocytosis [review: 32].

A small amount of neural inducing protein was isolated from the extracellular space between mesoderm and the neural plate, whereas proteoglycans from the extracellular space, isolated from the aqueous phase after phenol extraction, did not induce [33]. The proteoglycans did on the contrary prevent the autoneuralization of *Triturus alpestris* ectoderm cultured in Flickinger salt solution with 1.5 fold Na^+ ion concentration (Hildegard Tiedemann, unpublished experiments). The extracellular material did not contain RNA indicating little contamination from damaged cells. Duprat and Gualandris have shown that the extracellular material on the inner surface of the ectoderm is not implicated in neural determination [34]. The electric coupling of ectoderm and mesoderm which occurs 3–6 hr after combination of the two tissues [35] as well as the change of Na^+ and K^+ ion concentrations in induced ectoderm [36] may not be a prerequisite but a consequence of induction.

The induction of kidney tubules in meta-nephric mesenchyme in transfilter experiments depends on the other hand on direct cell to cell contact by cell processes penetrating the filter [37]. The cell to cell interaction leading to induction is dependent on protein glycosylation [38] and may be due to factors which are integrated into the plasma membrane or extracellular matrix proteins [for an example, 39]. Neural inducing protein factors have been separated from the soluble fraction of chicken embryo brain and retina by electrophoresis and isoelectric focusing [40] or by chromatography on DEAE-cellulose [15].

From *Xenopus* embryos neural inducing factors have been partially purified by Janeczek *et al.* [31, 41, 45 and unpublished experiments]. The factors

are found in oocytes and in gastrula stages in ribonucleoprotein particles of about 110 Å diameter, which are different from ribosomes or their subunits, in the high speed supernatant and in small vesicles. In addition a very small mesodem inducing activity (as shown by the induction of hindheads; 15, 18) was found in these fractions. A forehead (archencephalic) induction is shown in Fig. 2. The factors are inactivated by proteolytic enzymes. The neural inducing factor in the RNP-particles is a basic protein with an apparent molecular weight >70,000 for the undegraded factor. Smaller proteins with neural inducing activity arise probably by enzymatic cleavage of the larger ones.

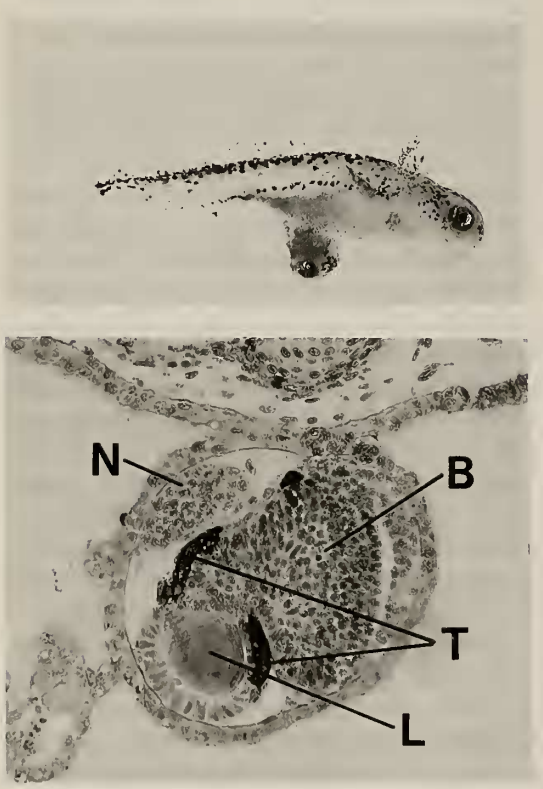


FIG. 2. A (upper). Forehead with eye induced on the ventral side of a *Triturus alpestris* larva by the implantation method. B (lower). Section through the forehead induction. L=lens; T=tapetum; B=brain; N=nose. The lumen of the induced nose is found in other serial sections of this induction.

The neural inducing factor in the supernatant has been partially purified by DEAE-cellulose chromatography [53] or by size exclusion HPLC [41]. The factor from gastrulae elutes at several size classes (Mr 16,000, Mr 35–50,000 and Mr 130,000–150,000) whereas the factor from oocytes is preferentially found in the largest size class. The factor is not inactivated after reduction with mercaptoethanol. Its molecular weight is not changed after reduction, but many contaminating proteins are shifted to smaller size. To take advantage of this fact the high speed supernatant from gastrulae was prepared in the presence of the protease inhibitors α_2-macroglobulin and leupeptin, reduced by mercaptoethanol and subjected to size exclusion HPLC. About 80–90% of the factor is then eluted at an apparent Mr of 100,000–150,000. When this protein fraction was subjected to SDS-polyacrylamide gel electrophoresis besides large proteins with an apparent Mr up to 150,000 also molecules of much smaller size were found. They obviously constitute a complex which is stable in 50% formic acid. Proteins of different size were then electroeluted from the gel and tested. About 20–30% of the neural inducing activity is found in proteins of an apparent molecular weight of 100,000–90,000, 70–80% of the activity in smaller proteins of Mr 15,000–25,000. Up to this step the smaller factor is purified about 800–1,000 fold. The experiments suggest that the larger factor could be a precursor of the smaller ones. The complex is not artificially formed in 50% formic acid. When the high speed supernatant was centrifuged on a sucrose gradient, most of the neural inducing activity was found in proteins larger than 100,000 Dalton.

It is possible, but has not been proven, that the factor in the RNP-particles is related to the supernatant factor. The factor in the ribonucleoprotein particles is, as was already mentioned, a basic protein, the factor complex in the supernatant, as the factor extracted from a fraction of small vesicles, an acidic protein (isoelectric point pH 5.5). Incubation with neuraminidase, hydrolysis with sulfuric acid under conditions where neuraminic acid is completely split from glycoproteins [44] or chemical deglycosylation with fluoromethansulfonic acid [Hoppe *et al.* unpublished experiments] did not change the isoelectric point. Treatment with phenol at 60°C does, however, convert a part

of the acidic neural inducing protein to a basic neural inducing protein perhaps by partially dissociating protein complexes.

Previous experiments have shown that the neuralizing factor is a maternal protein which is present in oocytes in ribonucleoprotein particles as well as in the supernatant in a masked biologically not active state [31, 44]. The neural inducing activity of the presumptive dorsal mesoderm increases from the morula stage onward [13, 46, 47]. This could depend on a partial activation of a maternal factor. The dorsal cortex has no inducing capacity [46]. The masked factors can artificially be activated by precipitation with ethanol, which denatures large protein complexes or by treatment with dissociating agents as urea, SDS or formic acid [31]. Whether the proteolytic cleavage of the precursor is related to the activation of the neuralizing factor is, however, not known. The precursor could be biologically inactive in the native state and treatment with dissociating agents like formic acid or SDS could lead to its activation. It is well known that for instance in enzymes regulatory domains can maintain a catalytic domain in an inactive state within a single peptide chain. In such molecules partial denaturation under dissociating conditions can have a similar effect as limited proteolysis (which eliminates the regulatory domain). Whether such an interaction between different domains of a single peptide chain exists in the large sized neural inducing factor is, however, not known. It is on the other hand not excluded (and may even be more likely) that other proteins which are associated with the factor keep the factor in a masked state and that the dissociation of the complex leads to its activation. The physiological process of demasking the factor(s) is unknown.

A small neural inducing activity has been found in germinal vesicles and in nuclei from later stages after activation with ethanol. Whether the factor in the nuclei differs from the factor(s) in the cytosolic fractions is not known [48]. Inducing factors are not integral proteins of plasma membranes [49].

Neural plates of *Triturus alpestris* induced by the underlying mesoderm acquire in turn neural inducing activity (homoiogenetic induction). This is correlated with the activation (and release) of a neuralizing factor from the neural plate and may suggest an autocrine mechanism. It has, however, to be proven whether the neuralizing factor from mesoderm is identical with the releasing factor or whether special releasing (and demasking) factors exist [50].

Both the factors from RNP-particles and from the cytosol remain fully active when they are covalently bound to bromocyano-Sepharose or bromocyano-Sephadex particles, which cannot be taken up by the ectoderm cells. Control experiments have shown that the inducing activity is not due to a release of the bound factors [51]. This suggested that a signal transduction mechanism is involved in neural induction. We could show that neural tissues are formed in isolated ectoderm of *Triturus alpestris* [52] and to a lesser extent in *Xenopus* ectoderm which in addition differentiates also to mesodermal tissues [53], when phorbolester (PMA = phorbolmyristate-acetate; TPA = tumor promoting agent) is added [52]. Phorbolester activates protein kinase C (PKC), which is assumed to be involved in the transduction of signals from the plasma membrane to the nucleus. The activity of proteinkinases has therefore been measured in isolated gastrula ectoderm induced with a neuralizing factor. Davids [53] has shown that the activities of proteinkinase C (or a related enzyme), which was measured with an enzyme-specific peptide substrate, as well as of c-AMP/c-GMP dependent kinases increase after induction. Addition of c-AMP or c-GMP or their mono- and dibutyryl derivatives to ectoderm does, however, not evoke neural differentiation [54]. It is therefore unlikely that the activation of c-AMP/c-GMP dependent kinases is the primary event in neural induction. Several proteins are more strongly phosphorylated in homogenates of induced ectoderm [53]. These proteins are also phosphorylated in homogenates of neural plates isolated from early neurula stages. The phosphorylation of 31 kD and 15 kD proteins seems to depend on PKC or a related enzyme. These phosphoproteins have first been detected 60 min after induction of isolated ectoderm with neuralizing factor. [Davids, unpublished experiments]. Their phosphorylation may not be the first event in neural induction, but may rather be part of a phosphory-

lation cascade. Otte *et al.* [55] have shown that protein kinase C is translocated to the plasma membrane after induction. Whereas phorbolesters are artificial activators of PKC's, the physiological activators are diacylglycerols or unsaturated fatty acids depending on the subspecies of the PKC's [review 56]. This may suggest that the breakdown of membrane phospholipids is involved in signal transduction after induction. Diacyglycerols and Inosintriphosphate are generated by phosphoinositide-specific phospholipase C (PLC), arachidonic acid is generated by phospholipase A_2. Experiments on signal transduction by adenylate cyclase have shown that the coupling of adenylate cyclase to effector occupied receptors is mediated by G-(GTP-binding) proteins. Adenylate cyclase activity is then terminated by GTP-ase activity intrinsic to the G-proteins. Non-hydrolyzable GTP-analogues as $GTP_\gamma S$ (guanosine-5′-O-thiotriphosphate) has therefore an intensifying effect. The finding that $GTP_\gamma S$ stimulates PLC activity have led to the assumption that the control of PLC occurs in a way analogous to adenylate cyclase [57, 58 review 59].

$GTP_\gamma S$ (1 μM) can evoke neural differentiation in gastrula ectoderm of *Triturus alpestris* (but not of *Xenopus laevis*; Loppnow-Blinde and Tiedemann, unpublished experiments). Li^+ ions which are known for many years [60, 61] to evoke neural and mesodermal differentiation in amphibian gastrula ectoderm have also been shown to interfere with the phosphoinositide cycle [62, 63]. This could suggest that phospholipase C is involved in the induction mechanism. The other enzyme of the phospholipid metabolism which could be involved, phospholipase A_2, is easily activated by disturbances of plasma membrane conformation. It is possible that such processes could be related to the so called "autoneuralization" effect.

It has to be stressed that absolutely no autoneuralization occurred under the conditions for the test of neuralizing factors.

Phorbolester can activate the Na^+/H^+ antiport system [64, 65]. Ectoderm from early gastrula stages of *Triturus alpestris* (but at the concentrations employed not ectoderm of *Xenopus*) forms neural tissues when N-(2-hydroxyethyl) piperazine-N-ethansulphonic acid (Hepes) in its protonated form is added to the medium as a buffer substance [66]. These and other observations led to the consideration that Hepes could lead to an export of H^+ from the ectoderm cells by an activation of the Na^+/H^+ antiport system. Ethyl-isopropyl-ameloride (100 μM) a potent and specific inhibitor of the Na^+/H^+ antiport [67], does, however, not inhibit the induction of *Triturus alpestris* ectoderm by the neuralizing factor. [Cragoe and Hildegard Tiedemann, unpublished experiments]. Similarly the action of growth factors is not inhibited by Ameloride derivatives in physiological bicarbonate buffer. These and other observations [reviewed in 68] suggest that a change of pH is probably not an intracellular messenger for neural induction or growth stimulation.

Concanavalin A, a lectin which binds especially to mannose residues in glycoproteins and Concanavalin A coupled to Sepharose evoke neural differentiation in gastrula ectoderm of *Triturus pyrrhogaster* [69] as well of *Xenopus laevis* [70] and of *Rana temporaria* [71, 71a]. The latter is only weakly induced by Con A-Sepharose [71]. Concanavalin could either bind to a cell surface receptor for the neuralizing factor or it could change the conformation of the plasma membrane after binding to distinct sites. Other lectins lead to a loss of neural competence [72]. Retinoic acid does not induce neural differentiation in gastrula ectoderm [Hildegard Tiedemann, unpublished experiments, 73]. The substance causes, however, microcephaly. It has been suggested that retinoic acid specifies regional differentiation of the central nervous system in amphibians [73] and in chicken the anterior-posterior axis during limb development [74]. The identification of nuclear receptors for retinoic acid in several tissues speaks strongly for its function as a physiological regulator, but its many teratogenic actions at a low concentration make it somewhat difficult to discriminate between these two possibilities.

A neural cell adhesion molecule [N-CAM, 75] is expressed during early neurogenesis in *Xenopus* [76]. Other neural specific proteins expressed after induction are neurofilaments and tetanus-toxin binding sites [77].

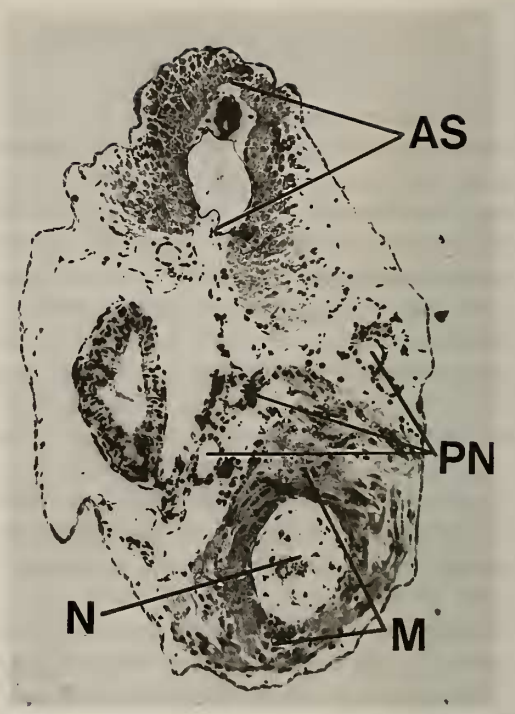
Induction of mesoderm and endoderm and the factors involved

The presumptive dorsal mesoderm has been regarded as the "organizer" of embryonic development. This should imply that this region is already determined to its fate in the fertilized egg. But when in 1962 Nakamura [78] isolated the presumptive mesoderm (the marginal zone) from different developmental stages of *Triturus pyrrhogaster,* the isolated mesoderm from very early stages did not differentiate into mesodermal tissues, its prospective fate. The marginal zone acquires its differentiation capacity in the morula stage. This demonstrated the epigenetic development of the "organizer" [79]. Hildegard Tiedemann [80] in 1965 observed that gastrula endoderm of *Triturus alpestris* when implanted into the blastocoel of early gastrula hosts induced in the ventral ectoderm mesenchymatic tails in about 20% of the cases. In 1967 Ogi [81, 82] combined isolated endoderm and ectoderm and obtained the induction of mesodermal tissues. He explained the formation of mesoderm as a result of regulation on the basis of two opposite animal-vegetal and vegetal-animal physiological gradients. The induction of mesodermal tissues in ectoderm explants which were combined with endoderm has been investigated in detail by Nieuwkoop and collaborators [83, 84] and Nakamura and collaborators [85]. Nakamura emphasized the importance of an animal-vegetal gradient, Nieuwkoop the induction of mesodermal tissues in ectoderm by the endoderm [86, 87]. Both views are certainly not mutually exclusive. Grunz and Tacke [88] have shown that the induction of mesoderm is not prevented by placing a Nucleopore filter between endoderm and ectoderm. Electronmicroscopy did rule out cell processes traversing the filter. The inducing effect is obviously mediated by diffusible factor(s). Dawid *et al.* [89] came to a similar conclusion. They observed that the appearance of a muscle specific marker was prevented by completely dissociating and dispersing *Xenopus* embryos during the period from early cleavage to early gastrula, a procedure that would dilute secreted inducing factors. Gurdon *et al.* [90] have concluded from dissection experiments that the "subequatorial" zone of the fertilized *Xenopus* egg contains all components for muscle gene activation. Because the boundaries of the subequatorial zone are not exactly defined, the zone could include some presumptive endoderm. It is, however, not excluded that active factors which are needed for the differentiation of mesodermal tissues are in the fertilized egg already localized in the vegetal most part of the marginal zone. Asashima [91] has investigated the inducing capacity of endoderm from different stages of *Triturus alpestris.* Endoderm taken from uncleaved eggs induces mesothel and blood cells in a low percentage, whereas endoderm from later stages in addition induced muscle, notochord and pronephric tubules. Blastula endoderm has the highest inducing activity. Thereafter the inducing activity declines. The increase may depend on the activation and release of masked factor(s). *Xenopus* endoderm induces mesoderm from the cleavage to the early gastrula stage [92].

The inducing capacities of the dorsal and the ventral endoderm differ. Boterenbrood and Nieuwkoop [93] have shown that the dorsal endoderm induces dorsal mesodermal tissues (notochord and somites) whereas the ventral endoderm induces more ventral mesodermal tissues (absence of notochord, no well arranged somites, blood cells). Experiments with cell lineage labels and region specific markers confirmed that the dorsovegetal material induces dorsal type mesoderm and ventrovegetal material ventral type mesoderm [94]. Yamada has already shown in 1940 [95] that organs which are formed from different presumptive mesodermal regions change to a more dorsal type (i.e. blood cells to nephric tubules or nephric tubules to somites) when the notochord anlage is added to the explants. This suggests that within the presumptive mesoderm a dorso-ventral gradient of (still unknown) regulatory factor(s) is established, which in addition to factors from the ventral and dorsal endoderm is involved in the subdivision of the mesoderm. Gurdon *et al.* have shown that in embryos which have just completed gastrulation α-skeletal and α-cardiac actin genes start to be transcribed in the somite region of the mesoderm and to a lesser extent in the ventral mesoderm, which possibly

gives rise to the heart [96]. Actin c-DNA probes have been used as mesoderm markers. The ability to react to inducing factors, the competence of the ectoderm, is limited to certain stages. The reason for this temporal limitation is not yet known. In *Triturus alpestris* [97] and to a lesser extent in *Xenopus laevis* [98], the loss of competence is delayed when the protein synthesis in the ectoderm is inhibited.

A factor which induces mesoderm and endoderm has been isolated from 9–11 days old chicken embryos by Tiedermann *et al.* [99–102]. The factor is protein in nature. The most efficient way for its separation from nucleic acids is the extraction with phenol [103]. The phenol procedure was developed because at that time it was thought that the factor could be RNA in nature. The phenol procedure has then been widely used for the preparation of RNA. The RNA did, however, not show inducing activity. The final purification of the factor was achieved by size exclusion and reversed phase HPLC. The acid stable factor, which is enriched about 10^6 times, has been called vegetalizing factor, because the tissues which are induced constitute the vegetal half of the embryo. On the basis of our earlier investigations the factor has recently been isolated in higher yield [Tiedemann *et al.* unpublished results]. The method employs extraction with acid ethanol, the final purification is achieved by four consecutive steps of reversed phase HPLC. The factor induces at a concentration of 0.5–1.0 ng/ml in about 50% of the cases mesoderm, including muscle. The apparent molecular mass of the factor determined by SDS-polyacrylamide-electrophoresis is about 25,000 Dalton and that of the biologically inactive subunits after reduction of disulfide bridges 13,000 Dalton, the isoelectric point about pH 8,0. By size exclusion chromatography in 50% formic acid an apparent molecular mass of 13,000 was found [102]. The dissociation into subunits may be caused by reduction of interchain or intrachain disulfide bonds by formic acid and conformational changes. The inducing activity is diminished after size exclusion HPLC in 50% formic acid. It is only partially restored after the removal of formic acid. The inducing activity is not diminished when the partially purified factor was incu-



A

B

Fig. 3. A. Mesoderm induced in a *Triturus alpestris* larva by the implantation method. Induced tissues: N = notochord; M = muscle; PN = pronephric tubules. AS = Axis system of the host larva. B. Section through a *Xenopus* explant with induced somites.

bated with formic acid. A mesoderm inducing factor (Mr 23,500) which was isolated by Smith *et al.* from the XTC (fibroblast) cell line of *Xenopus laevis* [105] has similar properties [104]. Another factor which has similar properties as the factor from chicken embryos has recently been isolated from calf kidney (Plessow and Davids, unpublished experiments). This suggests that the factor is an evolutionary conserved protein which may also have regulatory functions in later stages of embryogenesis, in adult differentation processes such as erythrocyte or cartilage differentiation or in regeneration processes. Asashima and coworkers have made the interesting observation that activin A, which is identical with the erythroid differentiation factor (EDF), has mesodem inducing activity at a low concentration [106].

The vegetalizing factor induces, depending on its concentration all kinds of mesodermal tissues. Endoderm seems preferentially to be induced at a very high concentration. At gradually lower concentrations pronephros, somites (Fig. 3), notochord and mesothelia are induced [107]. In addition to mesodermal and endodermal tissues cells with the typical appearance of primordial germ cells were observed in explants which were cultured for at least 20 days [108].

When tested at a very high concentration by the implantation method the vegetalizing factor causes an exovagination (not exogastrulation) of the gastrula (Fig. 4). Endoderm which had invaginated during gastrulation, reappears in the blastopore and spreads over the induced ectoderm. The exovagination is caused by a change of cell affinities [109] of the gastrula ectoderm induced to endoderm and mesodem. A similar effect has been observed after injection of XTC-cell factor into the blastocoel of *Xenopus embryos* [110].

The vegetalizing factor is in contrast to the neural inducing factor inactivated after covalent coupling to BrCN-sepharose or BrCN-sephadex [111]. The activity is completely recovered after degradation of the sephadex matrix with dextranase [112]. This suggests that the factor must be taken up by the cells to become biologically active. It does not exclude that cell surface receptors are involved.

A factor from guinea pig bone marrow which was partially purified by Yamada and Takata induces as the vegetalizing factor besides mesodermal also endodermal tissues [10, 113]. The histological identification of endodermal tissues is, however, difficult because endoderm differentiates late. The availability of endodermal

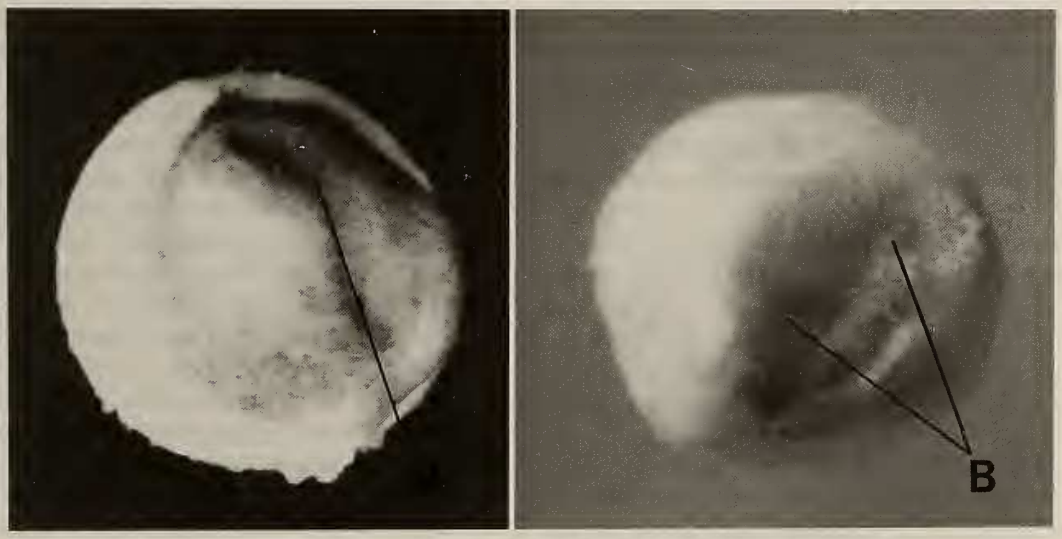


FIG. 4. Exovagination of *Triturus alpestris* embryos produced by the implantation of vegetalizing factor in high concentration into the blastocoel. The embryos are partially overspread by white migrating yolk-rich endoderm. A. N=small neural plate. From Kocher-Becker and Tiedemann, Science, 147: 167 (1965). Copyright 1965 by the AAAS. B. =rudiment of epidermis. A small rudimental neural plate was found in histological sections.

markers will therefore facilitate the detection of endoderm. Recently Rosa [114] has isolated mRNA's induced in *Xenopus* ectoderm by a partially purified XTC-cell factor. One of these RNA's encoding a homeodomain containing protein (Mix 1), which is expressed 20′ after the addition of an inducing factor (XTC-cell factor or bFGF and TGF-β in combination) to ectoderm, is found in the embryo mostly in the future endoderm. Jones *et al.* [115] have prepared a monoclonal antibody which reacts with tail-bud endodermal tissues to identify endoderm induced in ectoderm explants.

Besides from guinea pig bone marrow mesoderm inducing factors have been extracted and partially purified from liver [116] and from the carp swim bladder [117].

The vegetalizing factor is *in vivo* in part bound to an acidic proteoglycan [118] and in vitro binds to heparin-sepharose which was used by Born *et al.* for affinity chromatography of the factor [119]. It was therefore tempting to investigate whether heparin binding growth factors of the FGF (fibroblast growth factor) protein family induce mesodermal tissues. It could indeed be shown that a-(acidic) as well as b-(basic) FGF induce the formation of mesodermal tissues [120–123]. Like the vegetalizing factor from chicken embryos both FGF's induce at a high concentration somites and at lower concentrations endothelium lined vesicles which contain, besides some pycnotic cells, single cells with the typical appearance of immature blood cells [123]. Recombinant human b-FGF induces at higher concentrations besides skeletal muscle also heart muscle with its typical honeycomb like appearance, surrounded by a mesothelium lined pericardial cavity [124].

In *Xenopus* the determination of heart mesoderm occurs prior to the end of gastrulation. The heart mesoderm is located in the gastrula in the deep zone lateral to the head mesoderm and migrates laterally and ventrally to fuse in the ventral midline during the late neurula stage [review 125]. The deep dorsal endoderm seems to contribute to the specification of heart mesoderm, whereas the superficial pharyngeal endoderm may enhance heart morphogenesis during later stages [126]. It is possible that in *Xenopus* ectoderm explants endoderm, which is induced by b-FGF, undergoes regional differentiation and specifies the heart anlage. In urodeles (*Triturus alpestris*) no heart is formed when the endoderm is removed at the neural plate stage [127].

Notochord is not or very seldom induced by the FGF's in *Xenopus* ectoderm. The notochord anlage is the dorsal most part of the mesoderm. It has been suggested that FGF's induce preferentially ventral mesoderm [120]. The spectrum of tissues, which are induced depends also on the concentration of the factors, the species, and the test methods which are used. Recombinant b-FGF induces besides other mesodermal tissues also notochord in ectoderm explants of *Triturus alpestris*. Notochord is very rarely induced by the vegetalizing factor from chicken embryos in *Xenopus* ectoderm explants, but is induced at higher frequency when tested by the implantation method on *Triturus alpestris* gastrulae. Acidic and basic fibroblast growth factors show an amino acid sequence homology of 57%. To the FGF protein family belong also oncogene products and interleukins [review 128]. The protein products of the oncogenes int-2 and hst/ks (kfgf) have been shown to induce mesoderm with different potencies [129].

The vegetalizing and the fibroblast growth factors share heparin affinity but differ in other properties such as hydrophobicity, inactivation after reduction of disulfide bonds and molecular mass. In these properties the vegetalizing factor and the XTC-cell factor are more closely related to the transforming growth factors β. The transforming growth factors β stimulate phenotypic transformation (anchorage independent growth) of two cell lines, but their preferential action seems to be growth inhibition. Whether TGF-β stimulates or inhibits cell growth seems to depend on the entire set of growth factors acting on a cell [130, 131]. The promotion of angiogenesis by TGF-β seems to be mediated by monocytes which are attracted and stimulated to synthesize interleukin 1 [132]. The TGF-β family comprises genes with regulatory properties in embryogenesis, the β subunits of inhibin and the activins, substances which regulate the release of the follicle stimulating hormone [review 128]. The erythroid differentiation factor [EDF; 133] is identical to activin A, a homodimer

consisting of two β_A subunits [134]. The TGF-β family includes also the Vg1 gene, which was discovered by Weeks and Melton [135]. The m-RNA transcribed from this gene is uniformly distributed in the cytoplasm of immature *Xenopus* oocytes, but is then translocated to the vegetal half where it is localized as a crescent at the vegetal pole of mature oocytes [136].

Rosa *et al.* and Knöchel *et al.* have shown that transforming growth factors induce mesodermal tissues [122, 137, 138]. TGF-β1 and β2 induce at a concentration of 1 μg/ml in *Triturus alpestris* ectoderm in about 60% of the cases small endothelium lined cavities which contain immature blood cells as well mesenchyme and in elongated explants at one pole a dense blastema tissue and metameric strands of cells like lateral plate mesoderm, which in the distal part of the explant form large masses of endothelial (mesothelial) networks. The networks can form capillary like structures. Muscle and notochord are induced in *Xenopus* and *Triturus* ectoderm only by TGF-β_2. *Xenopus* explants were not induced by TGF-β_1 [137]. The TGF's or closely related factors induce in mammalian cell culture cartilage [139]. Asashima *et al.* [117] have recently shown that activin A (EDF) induces mesoderm at a low concentration. Activin A has a 40% sequence homology to TGF-β. Activins and inhibins bind as the vegetalizing factor to heparin-Sepharose [140]. The affinity of these factors to heparin is, however, lower as compared to the fibroblast growth factors. Binding to heparin depends on the native protein structure. Because the TGF's are extracted under dissociating conditions which change their protein conformation, it is not known whether the TGF's bind also to heparin.

The transforming growth factors are probably not identical with, but related to the vegetalizing factor and the XTC-cell factor. TGF-β and a mesoderm inducing factor in human blood platelets can be separated by size exclusion chromatography [Dau *et al.* unpublished experiments]. The growth factors must be applied to gastrula ectoderm in higher concentrations than the inducing factors for mesoderm induction.

The factors which determine endoderm and induce mesoderm in the embryo have not yet been definitively identified. Kirschner *et al.* [141] have found that a m-RNA which is present in *Xenopus* oocytes and newly transcribed in the neurula stage, codes for a protein that is 84% identical to human b-FGF. The recombinant protein which was expressed from the c-DNA, induces at 20–50 ng/ml muscle specific actin m-RNA. This protein may be a natural inducer. b-FGF like proteins have been enriched from *Xenopus* eggs and embryos by heparin-Sepharose affinity chromatography [141, 142]. The factor is extracted in higher yield in the presence of Chaps, a zwitterionic detergent [Tiedemann *et al.*, unpublished experiments] and may in part be bound to particulate structures. Slack *et al.* [143] have identified receptors for the fibroblast growth factor in *Xenopus* blastula ectoderm. Besides the 4,2 kb transcript coding for the b-FGF like protein, a smaller transcript of 1 kb has been found which represents an antisense transcript to part of the FGF gene. It codes for an evolutionary conserved protein with a hitherto unknown function [144]. In addition to a b-FGF-like factor mesoderm inducing factors which are not bound to heparin-sepharose are present in *Xenopus* embryos. So far we could not extract with acid ethanol from the early stages of amphibian embryos a mesoderm inducing factor with properties similar to the vegetalizing or the XTC-cell factor. This could be due to the low solubility of the crude proteins, or sequences homologous to these factors could be integrated into larger proteins with other properties.

That different factors induce mesoderm is not unexpected. The factors could either induce more dorsal or more ventral regions of the mesoderm. They may also interfere with different targets in signal transduction chains from the cell surface to the chromatin.

Gene activation and pattern formation in early embryogenesis

In experiments with the vegetalizing factor from chicken embryos Minuth and Grunz [145] have shown that the differentiation of liver is enhanced by preventing interactions between the induced cells by dissociation of the induced *Triturus* ectoderm for 20 hr before reassociation.

Mesodermal tissues were induced at a high percentage if the ectoderm was not dissociated. This suggests that not different threshold concentrations of one factor, but cell interactions, in which additional factors are involved, are needed for the induction of different mesodermal tissues. Other experiments support this view. A shift in the quality of the induced tissues from mostly endoderm (induced at a high concentration of vegetalizing factor) to muscle and notochord was observed when a protein fraction, which was separated during the purification of the factor, was added to the highly purified factor. The added protein fraction alone had no mesoderm inducing activity [146]. Additional factors seem also to be involved in the induction of mesoderm by TGF-β. Medium which was conditioned by TGF-β induced ectoderm enhances the inducing activity [138]. This suggests that additional factors are secreted, which are either synthesized or activated in gastrula ectoderm treated with TGF β.

This does, however, not imply that endoderm is generally induced first and that factors generated in the endoderm then induce mesodermal tissues. A gene or genes activated by an inducer could activate other genes in the same cell or in neighboring cells, so that a network of genes would be generated. In induced ectoderm explants a large variety of interactions would be possible depending on inducer concentration, time of inducer action and of geometry. This can explain that in explants a variety of tissues in different proportions are induced.

The factors for determination of the axis system of the embryo and for the induction of the neural anlage are at least in part of maternal origin. The position of the factors which determine endoderm and mesoderm in the oocyte depends on cytoplasmic movements after fertilization [147]. The vegetal most blastomeres play an important part in axis formation. Gimlich and Gerhart [149] have shown that after UV-irradiation of the egg, which impairs the formation of axial mesodermal and of neural structures, one to three cells of the vegetal most octet of blastomeres from non-irradiated embryos of the 64 cell stage can partially or completely reconstitute axis formation. The inducing factors have probably their highest concentration in these cells. Whether the maternal factor(s) which determine the endoderm act within the cell in which they are located, or by an autocrine mechanism on neighboring cells remains to be shown.

The mesoderm inducing factors are located in fertilized eggs and early embryos in a graded distribution [review 80, 87]. The precise localization of the factors and their mRNA's will, however, only be known when the genes coding for the factors have been isolated. It will then be possible to synthesize c-DNA's and after insertion into expression vectors the proteins, so that the distribution of the factors and their mRNA's can be mesured by immunofluorescence or hybridization methods. So far only a Xenopus b-FGF related gene has been isolated [141].

The areas in which the factors are located in the embryo are probably larger than the areas of the tissues which are determined by these factors. A small amount of a mesoderm inducer is found in the animal (ectodermal) cap [150]. It is likely that not only a vegetal-animal graded distribution of factors, but also an animal-vegetal distribution of so far unknown factors exists. Animal pole explants of *Xenopus* express epidermis specific antigens which are not expressed in the vegetal half. The information to express one of these antigens is present in the animal half before cleavage [151, 152].

It should be borne in mind that the factors can be masked so that their total concentration is not equal to the concentration of the biologically active factor(s) or that other substances could counteract the inducing factors. Furthermore as in *Drosophila,* factors which repress gene activities [153] could be involved. Thus the ratio of two factors could decide whether a gene is activated in a certain position in the embryo. One factor in a graded distribution could on the other hand activate more than one gene depending on different threshold concentrations of the factor. A concentration dependent activation of different genes has been observed for the *Drosophila* biocoid protein [154–157]. It is, however, unlikely that one and the same factor directly induces different tissues at different threshold concentrations. A number of evolutionary conserved genes including homologs

of *Drosophila* regulatory genes are transcribed in *Xenopus* oocytes and embryos. Their differential expression in the embryo is one of the earliest events leading to tissue differentiation. The distribution of regulatory gene products seems not to be confined to the borders of the germ layers which in later stages reflect the tissue borders [114, 158].

These regulatory genes include genes which specify proteins with homeotic domains [review 159; 24, 25, 114, 160, 161] as well proteins with finger domains [162–164]. Both domains bind to DNA sequences and are thought to act as transcription factors.

Differential cell affinities which develop in the embryo [165] and the differential distribution of molecules of the extracellular matrix will then guide the morphogenetic process.

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